Low-dose Ultraviolet B Rays Alter the mRNA Expression of the Circadian Clock Genes in Cultured Human Keratinocytes

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Current understanding of mammalian circadian rhythms suggests that they are regulated by light targeting signaling pathways in the hypothalamic suprachiasmatic nuclei. Recently, investigators have identified the existence of extraretinal photoreceptors and a potential role for the skin in this regulatory process has been implied. We demonstrated that mRNA of the circadian clock genes *Per1*, *Clock*, and *bmal1/mop3* are expressed in normal human cultured keratinocytes. Low-dose ultraviolet B rays initially downregulate all circadian clock genes and then induce altered expression of the genes in keratinocyte cell cultures. Ultraviolet light targeting superficial layers of skin (keratinocytes) may therefore contribute to circadian rhythm modulation.
Key words: bmal1/mops/circadian rhythm/Clock/Per.
J Invest Dermatol 119:1220–1223, 2002

Circadian rhythms are a fundamental property inherent in most organisms. They are associated with oscillations of a wide variety of biochemical, physiologic, and behavioral processes with an ≈ 24 h periodicity corresponding to night and day. These rhythms are modulated by an endogenous timekeeping mechanism. Much progress has now been made at the cellular and molecular levels in understanding this system. Elucidation of this intrinsic clock has been facilitated by the cloning of circadian genes (*Per*, *tim*, *Clock*, *bmal1/mop3*, and *Cry*) in several species, including *Neurospora*, *Drosophila*, and mice (Antoch *et al*, 1997; King *et al*, 1997; Sun *et al*, 1997; Tei *et al*, 1997; Gekakis *et al*, 1998; Hogenesch *et al*, 1998; Kume *et al*, 1999; van der Horst *et al*, 1999).

Circadian rhythms are primarily thought to be regulated through the retina with subsequent signaling pathways to the suprachiasmatic nuclei (Darlington *et al*, 1998; Shigeyoshi *et al*, 1997; Jin *et al*, 1999). These input structures serve as the principal circadian oscillators of the body. The trigger for circadian rhythms is believed to be light reaching the retina in the eye. The retina has therefore been considered the main detector for light in this system (Foster *et al*, 1989). The circadian clock is regulated by a feedback loop involving *clock* + *bmal1/mop3*, which form the positive transcriptional elements (Gekakis *et al*, 1998; Hogenesch *et al*, 1998), and a family of three *Per* genes (*Per1, Per2*, and *Per3*), *tim, Cry1*, and *Cry2*, which form the negative elements of this mammalian loop (Shigeyoshi *et al*, 1997; Darlington *et al*, 1998; Zylka *et al*, 1998).

Manuscript received December 11, 2000; revised February 7, 2002; accepted for publication August 24, 2002

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This model, however, is not so straightforward. Investigators have shown that mice lacking visual photoreceptors in the retina display circadian responses to light similar to those observed in normal mice (Foster *et al*, 1991). In addition, some blind patients maintain a circadian rhythm of plasma melatonin concentration, a substance normally suppressed by light (Czeisler *et al*, 1995). These findings have suggested the existence of an unidentified extraretinal photoreceptor within mammals.

Campbell and Murphy (1998) have demonstrated that light pulses to the popliteal region induce the phase shift in the circadian rhythms of the melatonin concentration and core body temperature in humans. The investigators suggested the skin may be a light-sensory organ and a modulator of circadian rhythms in humans. The results of these experiments drew great attention and, although a number of attempts to replicate and further investigate these findings were made, the overwhelming data could not support their conclusions (Lockley *et al*, 1998; Hebert *et al*, 1999; Lindblom *et al*, 2000). Most recently, Wright and Czeisler (2002) repeated this experiment and demonstrated they could not reset the circadian phase with bright light exposure behind the knees.

The picture, however, is still incomplete. Further evidence has indicated that clock genes are more widely distributed and not specifically expressed in the suprachiasmatic nuclei as initially believed (Albrecht *et al*, 1997; Welsh *et al*, 1995; Oishi *et al*, 1998). For example, circadian clock genes have been shown to be expressed in cultured cells (Rat-1 fibroblasts and H35 hepatoma cells; Balsalobre *et al*, 1998) and several peripheral organs in mice (Albrecht *et al*, 1997; King *et al*, 1997; Sun *et al*, 1997; Tei *et al*, 1997; Hogenesch *et al*, 1998; Oishi *et al*, 1998; Sangoram *et al*, 1998; Zylka *et al*, 1998). Circadian expression of clock genes has also been observed in the oral mucosa and skin of human subjects (Bjarnason *et al*, 2001).

In this study, we sought to determine if circadian clock genes were expressed in cultured human keratinocytes and if are they

could they be modulated by ultraviolet (UV)B. We demonstrate for the first time that circadian clock genes are expressed in human keratinocytes and that these genes can be regulated by UVB.

MATERIALS AND METHODS

Keratinocyte culture Normal human keratinocytes were obtained from neonatal foreskins as previously described with minor modification (Kondo *et al*, 1993). Briefly, the epidermal sheets were separated from the dermis after incubation in 1% dispase (Boehringer Mannheim, Humilton, Germany) at 4°C overnight. The epidermal cells were disaggregated by trypsinization and cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 100 U per ml penicillin G, 100 U per ml streptomycin, and 0.25 μg amphotericin B per ml. Two days later, the media was changed to keratinocyte serum-free media supplemented with bovine pituitary extracts and recombinant epidermal growth factor (Gibco BRL, Burlington, Ontario, Canada). The cells were then subcultured and maintained in keratinocyte serum-free media.

UVB irradiation Cells were irradiated with four fluorescent lamps (FS20T12-UVB, National Biological Corporation, Twinsburg, OH), which emit wavelengths between 280 and 320 nm, with a peak at 313 nm.These lamps specifically emit UVB and do not produce wavelengths outside of this spectrum. The irradiance was 0.36 mW per cm^2 at a target distance of 15 cm, measured by an IL-1400A radiometer, equipped with a SEL 240/UVB 1/TD UVB detector (International Light Inc., Montreal, Quebec, Canada). Cells were washed twice by prewarmed phosphate-buffered saline, and irradiated with UVB (10 mJ per cm^2) in the presence of 0.5 ml phosphate-buffered saline. Immediately, after UVB exposure, the phosphate-buffered saline was removed and the cells were cultured with keratinocyte serum-free media.

A second set of "sham" keratinocytes were prepared as controls for this experiment. These cells were cultured and subjected to the exact same procedures as the experimental cells. The "sham" cells, however, did not receive any UVB irradiation. Both the experimental and "sham" cells were stored in the dark, only being briefly exposed to visible light during the experimental procedures.

Semiquantitative measurement of RNA using reverse transcription–polymerase chain reaction (reverse transcription–PCR) Cells were harvested and every 4 h during the 72 h following UVB exposure. Total mRNA was extracted from the harvested experimental and "sham" cells by acid guanidinium thiocyanate–phenol–chloroform method. Reverse transcription–PCR was performed as previously described (Chomczynski and Sacchi, 1987) using the following primers:
5′-CTCCCATCTGGGGAGGAGGT-3′ and 5′-GGACCATCTCCAGGA GTCCA-3′, corresponding to nucleotides 4072–4091 and 4454–4435, respectively, for human *Per1 (RIGUI)* (accession no. AF022991);
5′-ACTATGGTGATTTCTCAGCCTGC-3′ and 5′-CTGTTGCTGAGAC TGATGTTGC-3′, corresponding to nucleotides 2347–2369 and 2835–2814, respectively, for human *Clock* (accession no. AF011568);
5′-GAACCAGACAATGAGGGGTGT-3′ and 5′-CCTTCCAGGACGTTG GCTAAA-3′, corresponding, respectively, to nucleotides 1276–1296 and 1712–1692 for human *bmal1/mop3* (accession no. AF044288).
Primer sets specific for human *G3PDH* were purchased from Clontech Laboratories (Palo Alto, CA). Specific cDNA obtained from reverse transcription was amplified using 10 pmol of each primer and 0.5 U of Taq DNA polymerase (Pharmacia Biotech, Piscataway, NJ). PCR signals for *G3PDH, Per, Clock*, and *bmal1/mop3* were obtained after 24, 28, 28, and 32 cycles, respectively. An aliquot (4 μl) of the PCR product was electrophoresed on a 1.6% agarose gel and visualized by ethidium bromide staining and UV illumination. After photographing the gel, relative amounts of PCR products were determined by scanning the negative films using a laser densitometer (LKB 2222–020, Ultroscan, KL, Pharmacia). Each experiment was performed at least four times. Representative data are included in this study.

RESULTS

Circadian clock genes are expressed in normal human keratinocytes PCR products for *Per1, Clock*, *bmal1/mop3*, and *G3PDH* were clearly detected in normal human keratinocytes using the specific primers. The size of four products coincided with the predicted amplified fragments (383 bp for *Per1,* 489 bp for *Clock*, 437 bp for *bmal1/mop3,* 984 bp for *G3PDH*; **Fig 1**). Furthermore, the expression levels of the clock genes and the housekeeping gene *G3PDH* were assessed using mRNA from "sham" cells every 4 h over the 72 h period. The expression level of each mRNA did not vary significantly from one time period to another (data not shown). This corresponds to previous reports demonstrating that mRNA expression of clock genes showed no change in cultured cells (Balsalobre et al, 1998).

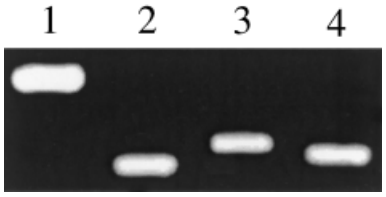

Figure 1. Expression of circadian clock genes mRNA in human cultured keratinocytes. PCR products for *Per1*, *Clock*, and *bmal1/mop3* are clearly detected, with the predicted bp sizes. *Lane 1*, *G3PDH (*983 bp); *lane 2*, *Per1* (382 bp); *lane 3*, *Clock* (488 bp); *lane 4*, *bmal1/mop3* (436 bp).

UVB induces expression of the clock genes The cultured keratinocytes were harvested every 4 h during the 72 h following UVB exposure. RNA was extracted from the cells and reverse transcription–PCR was performed to examine expression levels of the *Per1*, *Clock*, *bmal1/mop3*, and *G3PDH* genes.

UVB initially downregulated all circadian clock genes' expression. *Per1* showed an initial reduction in gene expression for 12 h following UVB irradiation. *bmal1/mop3* expression was suppressed for 20 h, and *Clock* for 24 h after UVB exposure.

After these time intervals, the genes recovered. *Per1* subsequently showed increased mRNA expression with a peak at 40 h. *Clock* expression showed a peak at 36 h with a trough at 48 h. The mRNA expression of *bmal1/mop3* showed no significant variation after the recovery (**Fig 2**). Interestingly, the *G3PDH* housekeeping gene showed no variation in mRNA expression (i.e., neither suppression or upregulation) following UVB exposure.

DISCUSSION

This study was prompted by Campbell and Murphy's (1998) observation that light exposure at the popliteal region could induce phase shifts of the circadian rhythms. A number of investigators have since disproved these findings (Lockley *et al*, 1998; Hebert *et al*, 1999; Lindblom *et al*, 2000), including Wright and Czeisler (2002) who recently repeated the experiment with bright light behind the knees and found that they could not reset the human circadian pacemaker. The exact mechanisms governing circadian rhythms in mammals and any contributing influences on these cycles, however, remain uncertain. Bjarnason *et al* (2001) found that clock genes were expressed in the oral mucosa and skin of human subjects and follows a circadian pattern of expression *in vivo*.

In this study, we first hoped to confirm that circadian clock genes were expressed in human skin. We demonstrated that the mRNA of the circadian clock genes (*Per1, Clock*, and *bmal1/mop3*) are expressed in normal cultured human keratinocytes.

We then sought to determine if light could modulate circadian clock genes. We chose UVB as it is known to modify genes in the skin (Kondo *et al*, 1993; Lee *et al*, 1997; Kang *et al*, 1998; Huang *et al*, 1999; Soriani *et al*, 1999). As well, UVB is a component of solar irradiation that mammals are exposed to during daylight hours

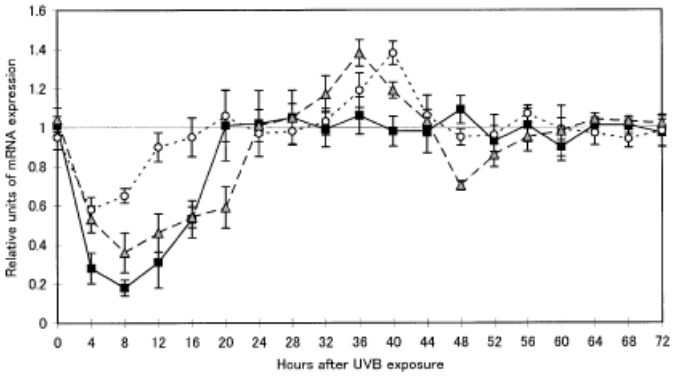

Figure 2. Alterations in the mRNA expression of circadian clock genes in cultured human keratinocytes following low-dose UVB exposure. By harvesting irradiated keratinocytes immediately following and every 4 h postirradiation, we obtain a 72 h temporal profile of mRNA expression of circadian clock genes in response to UVB exposure. The experiment was repeated four times and a mean gene expression value at each time point was calculated. Pre-exposure values for *Per1*, *Clock*, and *bmal1/mop3* gene expression were normalized to total mRNA and plotted on the y-axis (represented as 1 relative unit of mRNA expression). The relative mean values of gene expression measured post-UVB exposure compared with pre-exposure values are plotted around the y-axis. SEM is displayed for each value. No change in *G3PDH* housekeeping gene expression was noted pre-exposure and postexposure (data not plotted). Expression of *Per1* (---○---), *Clock* (---▲---), *bmal1/mop3* (---■---), and the *G3PDH* housekeeping gene were examined by semiquantitative reverse transcription–PCR and measured by a laser densitometer.

and has been shown to play a part in ocular circadian input (Brainard *et al*, 1994). The effects of visible light and UVA exposure on circadian gene expression in cultured keratinocytes were not examined in this experiment. Our results suggest that UVB induces altered expression of circadian clock gene mRNA in cultured keratinocytes. This was most pronounced with clock mRNA, which demonstrated a zenith at 36 h and a nadir at 48 h.

Unfortunately, the timeline of our experiment was too short to determine if the differential expression of the circadian genes induced by UVB light was truly rhythmic. The presence of the peak and trough noted with *clock* mRNA may suggest circadian cycling of these genes over time. Further experiments are necessary to characterize better the pattern of altered gene expression witnessed in this study.

The observations in this study are similar to those found by Balsalobre *et al* (1998) when they examined circadian gene expression in cultured fibroblasts. It is possible that the circadian genes are constitutionally expressed in keratinocytes and rhythmic expression is induced by UVB. Alternatively, UVB may synchronize already existing desynchronized gene cycles within these cell cultures. Unfortunately, the gene expression patterns of individual cells cannot be detected with the methods employed in our experiment. Which of these paradigms is correct, is difficult to say for certain.

In this study, the expression levels of the circadian clock genes were measured using semiquantitative reverse transcription–PCR. Whereas this is not considered the strongest tool for quantitative analysis and does not directly reflect the biologic significance of our observations, we feel that it is an appropriate method in this case. The identification of Per1, Clock, and Bmal1 proteins using monoclonal antibodies can be performed; however, the protein quantification has not been useful for studies involving clock genes. These proteins have not shown rhythmic activity in the suprachiasmatic nuclei or any other organs. mRNA, which tends to exhibit circadian rhythm expression, has therefore generally been used in quantitative studies.

The patterns of clock gene mRNA expression differ with genes and species. *Per1, Per2*, and *Per3* demonstrate clear circadian rhythms in mRNA expression in mice and rats (Sun *et al*, 1997; Tei *et al*, 1997; Oishi *et al*, 1998; Zylka *et al*, 1998). *bmal1/mop3* shows weakly rhythmical expression in mice (Hogenesch *et al*, 1998); however, a marked circadian rhythm can be observed in rats (Honma *et al*, 1998). The mRNA of *Clock* is rhythmically expressed in rats (Abe *et al*, 1998), but not in mice (Sun *et al*, 1997; Tei *et al*, 1997). Whereas in humans, cycling of *Clock* mRNA has not been demonstrated, our results showing a zenith at 36 h and a nadir at 48 h after UVB exposure suggest that *Clock* mRNA may be rhythmically expressed at least in response to UVB.

Solar irradiation reaching the earth's surface includes wavelengths ranging from the UV to the infrared spectra. Investigators have determined that blue light, found in the visible light range, upregulates *Per* gene expression (Shigeyoshi *et al*, 1997). The photoreceptor genes for blue light, *Cry1* and *Cry2*, have been cloned and CRY1 and CRY2 have been shown to regulate the clock feedback loop in mammals in a negative fashion (Emery *et al*, 1998; Kume *et al*, 1999; van der Horst *et al*, 1999).

UVB (10 mJ per cm^2) at 313 nm represent less than 1 minimal phototoxic dose in humans. After the keratinocytes were irradiated in this study, few dead cells were observed and cellular proliferation activity was comparable with that of the "sham" controls. In addition, the expression of *G3PDH* mRNA showed no change after UVB exposure (data not shown). It is therefore unlikely that the change in circadian gene expression we observed represents a phototoxic effect. Instead, our studies suggest that low-dose UVB has an initial downregulatory effect and then induces rhythmic expression of the circadian clock genes in normal human cultured keratinocytes.

Our studies have also demonstrated that the regulatory system, or feedback loop, in the circadian clock genes is functional in keratinocytes. In this system, CLOCK-BMAL1 heterodimers bind to E-boxes in the promoter region of *Per1* and drive its transcription. *Per1* gene products, on the other hand, inhibit transcriptional activity of the CLOCK-BMAL1 dimers (Sun *et al*, 1997; Gekakis *et al*, 1998). Our results revealed that the increase in *Per1* mRNA is preceded by increases in *Clock* mRNA. Furthermore, reduction in the *Clock* mRNA was observed following increased *Per1* mRNA expression. This implies *Per1* may inhibit *Clock* expression, consistent with the feedback loop as shown in many other models (Darlington *et al*, 1998; Shigeyoshi *et al*, 1997; Gekakis *et al*, 1998; Sangoram *et al*, 1998; Jin *et al*, 1999).

The retinohypothalamic tract is known to be an important input pathway to the suprachiasmatic nuclei (Moore and Lenn, 1972; Moore, 1973; Johnson *et al*, 1988). Visible light through the eyes is still regarded as the primary input for setting circadian rhythms. In this study, we chose to examine the effects of UVB on circadian clock gene expression because UVB is known to modify genes in the skin and is a component of solar irradiation that reaches the earth's surface during daylight hours. Furthermore, both UVB and UVA have already been shown to play a part in ocular circadian input in at least three or four mammalian species (Brainard *et al*, 1994).

Light stimuli to the retina, however, are not the only stimuli capable of resetting circadian rhythms. A phase-shift of the circadian rhythms is observed in response to locomotor activity and is blocked by lesions of the intergeniculate leaflet (Johnson *et al*, 1989; Reebs and Mrosovsky, 1989; Turek, 1989). Olfactory stimuli can facilitate the clock resetting by light in rats (Possidente *et al*, 1990; Goel *et al*, 1998; Amir *et al*, 1999). Our results suggest that UV light targeting superficial layers of skin, namely keratinocytes, may represent an alternate pathway for circadian rhythm modulation via changes in the expression of epidermal clock genes.

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